Field Evaluation of a Fluorogenic Probe-Based PCR Assay for Identification of a Visceral Leishmaniasis Gene Target

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ABSTRACT

*Visceral leishmaniasis (VL) is a potentially fatal disease caused primarily by **Leishmania donovani** complex species in Old World endemic regions. Pathogenesis requires a virulent factor transcribed by a gene family designated A2. An A2 sequence specific fluorogenic probe hydrolysis (TaqMan) PCR assay was developed on a field-deployable assay platform for real-time screening of the sand fly vector for VL causative agents. Laboratory-based assay optimization and specificity testing were conducted with total nucleic acid extracted from **L. donovani**-infected **Phlebotomus alexandri**, the primary vector. Cross-reactivity did not occur when tested against total nucleic acid extract of **L. tropica**-infected **P. sergenti, L. major**-infected **P. papatasi**, clinically relevant organisms, diverse viral and bacterial species, vector and human genomic DNA. Field evaluations were conducted in south central Iraq with sand fly pools screened by a previously established **Leishmania** universal PCR assay. The **Leishmania** universal assay was field-formatted (lyophilized) prior to deploying and upon field-evaluation against cold chain maintained wet reagents results were 98% (55/56) concordant. Of 86 **Leishmania**-positive sand fly pools, six were identified as A2 positive with a field-formatted visceral genotype specific assay.*

Introduction

The disease group leishmaniasises are caused by obligate intracellular parasites of the genus *Leishmania*. *Leishmania donovani* complex species are pathogenic agents of potentially fatal visceral leishmaniasis (VL) (1, 2). These protozoa exist as free-living promastigotes in the gut lumen of the sand fly vector (Old World genus *Phlebotomus*) and when transmitted to vertebrate host develop into amastigotes that proliferate within the macrophage phagolysosome. It has been shown that a *L. donovani* stage-specific gene family, designated A2, encodes protein required for amastigote development, which can ultimately be expressed in humans as VL (3, 4, 5). The leishmaniasises are also expressed in nonfatal forms, cutaneous and mucocutaneous. Old World visceral leishmaniasis, or kala-azar, is caused by *Leishmania donovani* complex species that include, *L. donovani* and *L. infantum*, and while *L. tropica* is typically a cutaneous leishmaniasis causative agent the virulence of this species can be manifested in the VL form of the disease (6).

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Iraq represents one of the most leishmaniasis prevalent regions globally (1). In the Nasiriyah area of south central Iraq the cutaneous forms of the disease are so common that cases usually go unreported (7). Clinicians in An Nasiriyah estimate nearly 1000 confirmed cases of VL each year for the past several years (7). Clinical laboratory methods of VL diagnoses are by microscopic identification of infected tissues, *in vitro* culture, or animal inoculation; identification of *Leishmania* DNA in infected tissue samples; and immunoassay (8). Immunochromatographic strip assays for the detection of antibodies to *L. donovani* in human serum are easily transported and require minimal training for use (9). An FDA approved kit is currently distributed to Iraqi clinicians through the World Health Organization and Coalition Provincial Authority, Baghdad, Iraq. Treatment of VL is an arduous process for the patient, requires specialized training for administration, and the expense untenable for many health care facilities (1, 2). Prevention is paramount in the control of the disease.

Surveillance of leishmaniasis causative agents requires deployable assays specific to pathogenic species and strains of *Leishmania*. Efficacious surveillance of VL causative agents allows timely assessments of transmission risk and time-critical implementation of personal protection measures and appropriate vector control responses. While the assay system described in the paper is not currently approved for clinical diagnostics, preliminary data suggest that it will prove a deployable method for rapid, sensitive, and specific identification of the visceral leishmaniasis causative genotype in sand flies. To validate the efficacy of our deployable system, field-formatted *Leishmania* universal (LEIS) and *Leishmania* visceral genotype specific (LVL) PCR assays were deployed to a visceral leishmaniasis endemic area in south central Iraq, September 2003. The study site was Tallil Air Base.

Materials and Methods

Primer and probe design

Visceral leishmaniasis genotype specific primer and probe sequences were designed by targeting A2 gene family loci and aligning homologous regions within *Leishmania donovani* complex species that excluded species from *L. tropica, L. major, L. aethiopica,* and *L. mexicana* complexes. Alignments were compared visually using the Clustal algorithm (10) in the MegAlign program of DNA Star software (Perkin Elmer, Norwalk, Conn.) [11]. Maximally conserved oligonucleotide sequences were chosen from *L. donovani* and *L. infantum* genomes downloaded from Genebank. *Leishmania tropica, L. major, L. aethiopica, and L. mexicana* complex species genomic sequences were aligned and visually evaluated to validate heterology with A2 primer and probe sequences. Genotype A2 specific primer and probe sequences can be requested through the corresponding author. The probe was a dual fluorogenic label design with a 5' reporter dye, 6-carboxyfluorescein (FAM), and 3' quencher dye, 6-carboxytetramethylrhodamine (TAMRA) [12].

Genotype A2 specific primers and probes sequence heterology with genomic sequences of closely related species through diverse genera were validated by BLAST database search (BLAST, Madison, Wisconsin) [13]. Melting temperatures were quantified and the absence of significant primer dimerizations and secondary structure (hairpin) formations were confirmed with PrimerExpress software (PE Applied Biosystems, Foster City, Calif.). Primers and probes were synthesized and quality control conducted commercially (Synthetic Genetics, San Diego, Calif.). Primers and probe were designated Leishmaniasis Visceral (LVL). A previously established *Leishmania* universal assay (LEIS) was obtained through collaboration with the Infectious Disease Clinic, Walter Reed Army Medical Center, District of Columbia and Walter Reed Army Institute for Research, Silver Spring, MD (14).

Sand Fly Pools

Over a five month period (April through August 2003) prior to this study, US Air Force and Army entomologists captured sand flies in battery powered light traps deployed at dusk within the Tallil Air Base perimeter at locations densely inhabited military personnel and uninhabited control locations. On the following morning, female sand flies were sorted into pools of 5-15, and preserved in ethanol. Total nucleic acid extract was prepared and screened for *Leishmania* spp by LightCycler-based PCR (Idaho Technology Incorporated, Salt Lake City, Utah; www.idahotech.com) (15) utilizing wet LEIS reagents. To evaluate the efficacy of the field-deployable assay system a blind test panels nucleic acid extracts was prepared from field-caught sand fly pools that had tested positive by LEIS wet reagent analyses (Table 1, 2).

DNA preparation

Total nucleic acid extracts were prepared with a commercially available, "off the shelf", thermo-stable, preformatted viral RNA purification kit, QIAamp viral RNA mini kit (QIAGEN, Valencia, California). Sand fly pools were homogenized in 560 µl AVL Buffer/Carrier RNA component of the kit with sterile, RNase free pestles and 1.5 ml tubes. Homogenate was cleared by centrifugation on a table-top centrifuge at 12,000 rpm for 60 seconds and total nucleic acid extract prepared following the manufacture's spin protocol. Each extract was suspended in 60 µl of elution buffer.

Reaction conditions

Wet *Leishmania* universal (LEIS) and visceral genotype specific (LVL) PCR assays were field-formatted, lyophilized, by a proprietary process [Idaho Technology Incorporated, Salt Lake City, Utah; www.idahotech.com]. Field analyses was conducted on field-durable, real-time, fluorimetric PCR/RT-PCR, thermocyling instrumentation - Ruggedized Advanced Pathogen Identification Device (R.A.P.I.D.) [Idaho Technology Incorporated, Salt Lake City, Utah; www.idahotech.com] with lyophilized proprietary master mix components (Idaho Technology Incorporated, Salt Lake City, Utah). Master mix reaction solution was prepared by adding 40 µl of PCR grade water to lyophilized master mix reagent and dispensing18 µl volumes into optical capillary tubes. To each capillary, 2 µl of RNA extract added from specimens or 2 µl PCR grade water for no template controls (NTC). Capillaries were placed in tabletop centrifuge and spun for 2-3 seconds at 3000 rpm to drive the reaction mixture to the bottom of the capillary. *Leishmania* universal and LVL assay lyophilized master mixes contained *L. donovani* genomic DNA template and was prepared by adding 40 µl of PCR grade water.

A standardized PCR thermal cycling protocol was established that consisted of an initial DNA denaturation step at 94°C for 2 minutes, and PCR for 45 cycles at 94°C for 0 seconds of template denaturation and 60°C for 20 seconds of combined annealing and primer extension. A single data point at the end of each annealing-extension cycle was collected and reported as TaqMan probe fluorescence released by 5'-nuclease activity during primer extension. Fluorimeter gains were set at 8-2-2 on channels 1, 2, 3 respectively. Protocols for both LEIS and LVL assays were identical. The criterion for a positive result was a significant increase in fluorescence over background levels as defined by an algorithm provided in the R.A.P.I.D analytical software (Roche Molecular Biochemicals, Indianapolis, Ind.).

Results

Prior to field evaluation LVL wet PCR assay conditions were optimized to 0.10 pg (3 genomic equivalents) of *Leishmania donovani* genomic DNA (Table 1). Specificity testing was conducted with Old and New World *Leishmania* species, skin scrape preparations from ten cutaneous leishmaniasis patients, blood samples from eight malaria patients, clinically significant viral and bacterial species representing diverse species and strains, and human genomic DNA (Table 2). Testing with *Leishmania* infected vector species did not demonstrate cross-reactivity or inhibition of the PCR. All cross-reactivity test results displayed no detectable fluorescence above background with the exception of *Streptococcus equi*, a critical threshold slightly above background fluorescence (CT = 42) was observed upon testing of a single sample. Fluorescence was not at a level above background to consider the *Streptococcus equi* as a cross-reacting species but validation testing is planned. Laboratory-based optimization and specificity testing were conducted with LVL wet reagents on RAPID instrumentation. Field-formatted LEIS and LVL assays were deployed for evaluation. The LEIS assay when tested against cold chain maintained wet reagents results were 98% (55/56) concordant (Table 3). Of 86 *Leishmania*-positive sand fly pools, six were A2 positive (Table 4).

Discussion

In mid-July 2003 a team of US Air Force and US Army entomologists conducting *Leishmania* surveillance at Tallil Air Base, south central Iraq, expressed concern of a potential leishmaniasis outbreak situation. In response, we designed visceral leishmaniasis (VL) pathogen genotype specific PCR assay (LVL) primers and probe and conducted assay optimization and cross-reactivity testing through a joint AFIOH, WRAIR, USAMRIID, CHPPM-W effort. Field-formatting (lyophilization) of LVL and *Leishmania* universal (LEIS) assays was conducted by a corporate partner, Idaho Technology Incorporated, Salt Lake City, Utah. By late August, we deployed a developmental phase surveillance system for screening sand flies for *Leishmania* spp and identification of VL causative agents. Field-formatted master mix reagents and positive control were easily transported, field-sustainable, and only required hydration and addition of sample template prior to PCR (16, 17, 18). Wet and lyophilized LEIS assay results were concordant in blind testing and the LVL assay showed that vector-borne pathogens for VL were a significant threat.

These data supported force protection measures previously implemented by the entomologist team – stringent personal protection and vector control. Preliminary reservoir surveillance data of the primary reservoirs, rodents and canids (dogs and foxes), indicate that rodents are significant in the *Leishmania* transmission cycle while canids are not. Studies are planned to determine if rodent control will break the transmission cycle.

Additional testing of the LVL assay is planned to determine the efficacy of A2 gene family derived primer and probe sequences as a universal VL genotype target site. That *L. donovani* promastigote-to-amastigote development, and VL pathogenesis, requires an A2 gene family encoded factor defines this protein as fundamental to the life cycle of *L. donavani* and pathogenesis of VL. This suggests conservation in A2 gene family sequence homology across VL causative agents. Continued LVL specificity testing with diverse strains of *L. donovani*, *L. infantum*, and *L. archibaldi* as well as New World visceral leishmaniasis causative agent, *L. chagasi*, is planned. Moreover, testing with *L. tropica* strains implicated as causative agents in the visceral form of leishmaniasis will yield valuable LVL specificity data and potentially provide insight into the pathogenesis of the leishmaniasises.

The LEIS and LVL field-formatted real-time PCR assays have potential as an aid in human diagnostics. The deployable assay system provides an advantage over conventional diagnostic instrumentation especially in areas were clinical laboratory facilities are not available. Immunochromatographic strip assays provide deployable testing capability however require refrigeration. Moreover, immunoassays can potentially produce false negative results in immunologically immature or immunocompromised leishmaniasis patients and false positive results in individuals whom have developed antileishmanial antibody in response to a previous infection (19).

This study demonstrated the operational utility of a deployable DNA probe-based assay platform for the surveillance of visceral leishmaniasis causative agents in sand flies and promising aid to clinical diagnostics. The assay system may yet have an additional operational application in *Leishmania* surveillance as rotating coalition forces return from southwest Asia to non-endemic regions.

Reference to trade name, vendor, proprietary product or specific equipment is not an endorsement, a guarantee or a warranty by the Department of the Defense, Army or Air Force, and does not imply an approval to the exclusion of other products or vendors that also may be suitable. The conclusions and opinions expressed in this document are those of the authors. They do not reflect the official position of the U.S. Government, Department of Defense, Joint Program Office for Chemical and Biological Defense, U.S. Air Force, U.S. Army, or the Association of Military Surgeons of the United States.

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Table 1 LVL PCR assay sensitivity

Genomic Quantity	Genomic Equivalents	CT
L. donovani 1.00 ng	2.90E+04	23.32
L. donovani 1.00 ng	2.90E+04	22.91
L. donovani 0.10 ng	2.90E+03	26.84
L. donovani 0.10 ng	2.90E+03	26.08
L. donovani 0.01 ng	2.90E+02	29.57
L. donovani 0.01 ng	2.90E+02	30.08
L. donovani 1.00 pg	2.90E+01	33
L. donovani 1.00 pg	2.90E+01	33.6
L. donovani 0.10 pg	2.90E+00	38.47
L. donovani 0.10 pg	2.90E+00	35.6
L. donovani 0.01 pg	0.00E+00	0
L. donovani 0.01 pg	0.00E+00	0

CT: critical threshold

Table 2 LVL PCR assay specificity

Species	LVL
Leishmania donovani	+
Leishmania tropica	-
Leishmania major	-
Leishmania mexicana	-
Leishmania braziliensis	-
Leishmania guyanensis	-
Leishmania amazonensis	-
Cutaneous leishmaniasis CS1 - CS10	-
Malaria Vivax CS1 - CS8	-
West Nile Virus 613ca	-
West Nile Virus 1278	-
Dengue virus serotype 1 CS1 - CS4	-
Dengue virus serotype 2 CS1 -CS6	-
Hanta virus	-
Leptospira borgpeternenii	-
Leptospira interrogans	-
Leptospira biflexia	-
Leptospira icterohaemmorrhagiae	-
Leptospira patoc-1	-
Rickettsia rickettsia	-
Ehrlichia chaffeensis pD2	-

Table 2 LVL PCR assay specificity (continued)

Species	LVL
Streptococcus . pyogenes	-
Staphylococcus agalactica	-
Staphylococcus. aureus	-
Neisseria meningitidis	-
Salmonella typhi	-
Brucella melitensis	-
E. coli O157	-
Homo sapien genomic DNA	-

Table 3 Conventional vs field-formatted LEIS PCR assay results

TAML Sample ID	Conventional LEIS PCR Assay Sreening (CT)	Conventional LEIS PCR Assay Confirmation (CT)	Field-Formatted LEIS PCR Assay Screening (CT)
888-001f			
1117-001f	29.83	29.98-29.13	28.91
1094-001aa			
1094-001c			
1120-001a	29.24	33.49-35.38	36.33
1121-001b	23.09	27.06-23.60	21.60
1094-001d			
1124-001c	27.30	28.56-27.97	25.96
1101-001b			
1129-001n			
1125-001b	21.09	31.34-19.85	18.56
1129-001d	27.08	26.11-26.99	24.83
1129-001x	27.13	26.35-26.48	24.35
1129-001o			
1129-001y	26.50	26.34-25.18	24.80
1129-001r			
1135-001c			
1139-001n	27.71	26.56-26.98	26.06
1220-001a	28.96	31.64-30.91	31.56
786-001c	28.46	29.53-28.25	27.70

786-001f	28.14	29.87-27.84	26.58
1135-001d			
789-001e	25.41	23.83-24.62	23.04
1139-001g			
1139-001h			
789-001l	24.93	25.63-25.59	23.26
1178-001d			
1214-001c	24.07	24.47-24.49	22.79
1214-001d	27.45	26.78-25.45	24.70
1178-001e			
1219-001a	23.31	20.30-21.45	18.56
1178-001f			
1194-001a			
1240-001a	22.12	21.52-23.16	19.91
1241-001a	28.04	27.49-27.70	24.36
1242-001a	24.14	23.43-24.21	21.92
1195-001a			
1225-001a			
1245-001c	29.28	29.48-30.90	28.94
1225-001b			
1248-001d	28.94	27.27-26.72	25.51
1251-001b	29.00	29.58-29.07	27.77
1238-001a			

1238-001b			
1252-001a	22.32	25.18-22.68	21.47
1268-001a			
1268-001d			
1252-001c	27.70	26.93-26.83	26.35
1255-001a	25.72	25.87-29.45	22.33
786-001e			
789-001a			
888-001d			35.20
888-001e			
1268-001b	18.66	20.42-18.50	17.25
1268-001c	22.63	22.31-23.63	19.89
1272-001a	22.11	22.00-21.74	19.35

CT: critical threshold

Table 4 Conventional LEIS vs field-formatted LVL PCR assay results

TAML Sample ID	Conventional LEIS PCR Assay Screening (CT)	Conventional LEIS PCR Assay Confirmation (CT)	Field-Formatted LVL PCR Assay Screening (CT)
1019-001c	31.79	32.9->36	0
754-001a	>36	>36->36	0
756-001c	>36	33.01->36	0
984-001a	>36	>36	0
901-001c	33.91	>36->36	0
901-001l	28.73	31.08-30.54	0
965-001a	24.21	27.03-26.38	0
965-001e	25.45	26.23-26.11	0
965-001g	31.03	32.38-32.01	0
965-001j	25.57	27.93-26.11	0
965-001k	30.29	32.32-32.23	0
1007-001d	26.47	27.04-26.89	0
1032-001g	>36	>36->36	0
1043-001b	24.71	24.88-24.39	0
1043-001c	33.10	33.02-32.68	0
1044-001a	21.59	21.78-21.57	0
1045-001c	28.57	29.76-29.45	0
1050-001a	23.30	25.86-25.08	0
1051-001c	24.82	26.6-26.27	0
1051-001d	23.31	25.16-24.88	0

1057-001a	28.31	27.54-27.72	0
1061-001a	28.86	29.29-29.71	0
1061-001b	>36	34.11->36	32.26
1061-001f	33.49	31.99-33.28	0
1071-001a	28.80	28.49-29.11	0
1074-001a	33.33	>36-Neg	0
1075-001b	32.05	28.41-Neg	0
1078-001b	29.57	>36->36	0
1079-001e	32.34	33.07-33.17	0
1097-001a	23.15	22.80-22.86	26.08
1103-001a	31.82	>36-35.7	0
787-001d	31.83	31.78-32.50	0
787-001e	>36	19.66-20.01	0
787-001g	19.47	26.16-26.02	0
788-001a	25.56	>36->36	0
884-001d	>36	>36->36	0
884-001f	34.26	20.84-20.61	0
884-001g	20.51	21.12-21.88	25.42
950-001p	27.93	28.35-27.75	0
1107-001a	28.03	28.47-28.42	0
1112-001b	26.63	26.87-27.55	0
1109-001c	25.86	27.01-26.28	0
808-001a	29.00	28.74-29.14	36.91
811-001a	25.18	25.47-25.57	0

812-001a	28.22	28.30-27.80	0
817-001a	23.78	23.73-24.51	0
817-001b	>36.00	35.72->36	0
819-001c	33.56	>36-Neg	0
1122-001c	30.10	29.18-31.43	0
1129-001q	35.69	33.72-33.36	0
1129-001v	34.18	>36-34.28	0
1139-001L	34.63	33.08-32.82	0
1141-001d	30.70	28.91-29.95	32.8
1141-001h	31.62	29.54-29.04	0
1172-001a	35.14	33.33-33.29	0
1174-001b	33.00	32.90-33.02	0
1180-001a	33.42	32.45-34.29	0
1220-001b	34.23	34.07-33.96	0
789-001b	30.43	30.62-30.47	0
789-001j	31.61	31.89-31.56	0
1214-001b	34.08	35.19-33.81	0
1214-001e	32.45	31.94-32.09	0
1214-001h	30.11	29.45-29.32	0
1214-001j	30.90	31.09-31.24	0
1216-001c	30.31	31.44-30.48	34.43
1238-001d	30.65	30.83-30.56	0
1239-001b	31.97	31.90-31.83	0
1239-001c	32.15	32.13-32.39	0

1243-001a	>36	35.48-32.15	0
1245-001e	33.01	32.82-35.56	0
1247-001a	>36	34.18->36	0
1248-001e	31.87	32.85-31.99	0
1249-001b	32.67	32.90-33.08	0
1250-001d	30.34	30.03-30.32	0
1251-001a	32.76	33.03-34.30	0
1251-001c	31.94	31.76-31.83	0
1253-001b	35.76	32.85-33.54	0
1253-001e	>36	33.97-35.78	0
1254-001a	>36	34.11-34.15	0
1257-001a	32.69	32.03-32.20	0
1268-001c	22.63	22.31-23.63	0
1272-001a	22.11	22.00-21.74	0

CT: critical threshold

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SYMPOSIA DISCUSSION - PAPER 23

Authors Name: Mr McAvin (US)

Discussor's Name: Capt (USN) Campbell (US)

Question:
There are published reports of the "Visceralizing" Cutaneous Leishmaniasis (L. Tropica) in troops returning from the first Persian Gulf War.

Given that you have shown your visceral leishmaniasis probe to specifically not recognize L. Tropica, are you concerned that your probe might miss (not detect) L. Tropica that may be capable of causing the potentially lethal, visceral form of the disease?

Author's Reply:
Yes specimens have been requested from WRAIR to determine if the LVL assay is capable of identifying viserotropic L. Tropica.